Aquatic Toxicology 222 (2020) 105477

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aqtox

Effects of tris (2-chloroethyl) phosphate (TCEP) on growth, reproduction and gene transcription in the protozoan *Tetrahymena thermophila*

Hui Hao[a], Siliang Yuan[a], Shiyang Cheng[a], Qian Sun[a,*], John P. Giesy[b,c], Chunsheng Liu[a,*]

[a] Hubei Provincial Engineering Laboratory for Pond Aquaculture, College of Fisheries, Huazhong Agricultural University, Wuhan, 430070, China
[b] Department of Veterinary Biomedical Sciences and Toxicology Centre, University of Saskatchewan, Saskatoon, Saskatchewan S7N5B3, Canada
[c] Department of Environmental Science, Baylor University, Waco, TX, United States

ARTICLE INFO

Keywords:
TCEP
Tetrahymena thermophila
Growth and reproduction
Exposure
Flame retardant
Invertebrate

ABSTRACT

As a typical organophosphorus flame retardant, tris (2-chloroethyl) phosphate (TCEP) has been widely detected in various environmental media. Toxicity of TCEP to vertebrates have been investigated, but potential effects on lower trophic level species were unknown to date. In this study, toxic effects and molecular mechanisms of toxic actions of TCEP on the aquatic protozoan *Tetrahymena thermophila* were evaluated by use of phenotypic observations, transcriptome sequencing analysis and real-time quantitative PCR detection. Exposure to 0.044, 0.411 or 4.26 mg/L TCEP for 5 days decreased the theoretical population, cell viability, number of cilia and cell size of *Tetrahymena thermophila* in a time- and dose-dependent manner. Meanwhile, RNA-Seq analysis indicated that exposure to 4.26 mg/L TCEP significantly changed expression of 2932 genes (up-regulation: 1228; down-regulation: 1704). Of these, expressions of 9, 10 and 17 genes that were enriched in soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) interaction in vesicular transport, proteasome and endocytosis pathway respectively were down-regulated. Data collected during this study suggested that exposure to high concentrations of TCEP might affect growth and reproduction of *Tetrahymena thermophila* through down-regulating transcriptional levels of genes encoding proteins associated with vesicle trafficking, proteasome and endocytosis.

1. Introduction

Flame retardants are additive materials with anti-combustion properties, which are primarily used in building materials, electronics, furniture, textiles, and plastic packaging materials. With rapid development of industry and urban construction, applications of polymer materials have become quite extensive, such that fire caused by polymer materials poses threats to public safety and human life. Therefore, development and application of flame retardants have attracted attention (Marklund et al., 2003; Andresen et al., 2004). In recent years, polybrominated diphenyl ethers (PBDEs) used as brominated flame retardants (BFRs) have gradually been banned, which resulted in switching to substitute products, such as organophosphate flame retardants (OPFRs) (Van der Veen and de Boer, 2012; Betts and Kellyn, 2008). Previous statistics showed that consumption of OPFRs in Europe reached 85,000 tons in 2005 (European Flame Retardants Association, 2007). In 2007, China accounted for 35 % of global production of OPFRs (> 70,000 tons) (Reemtsma et al., 2008; Wang et al., 2010). In addition, in 2011 global consumption of OPFRs was 500,000 tons (Van der Veen and de Boer, 2012; Wang et al., 2015). Tris (2-chloroethyl) phosphate (TCEP) was one of the OPFRs employed. It was reported that in 1989, worldwide production of TCEP was about 9000 tons, but declined to < 4000 tons in 1997 (World Health Organization, 1998). Due to considerations of occupational health and public safety, production of TCEP was banned in Europe and global production of TCEP decreased (Leisewitz et al., 2001). In 2006, production of TCEP in the United States was 227–454 tons (Van der Veen and de Boer, 2012).

Due to the fact that they are added to polymers rather than being chemically bound, most OPFRs can be readily released into the environment (Rodríguez et al., 2006). Results of previous studies indicated that OPFRs were often detected in a variety of environmental matrices and biota (Meeker et al., 2013; Sundkvist et al., 2010). As a representative OPFR, TCEP is commonly found in air, surface water, drinking water, sediment, dust and biota. For example, analysis of indoor air from 17 domestic and occupational environments revealed that concentrations of TCEP ranged between 0.4 and 730 ng/m^3 (Marklund et al., 2005). In Sweden, concentrations of TCEP in air from a computer

* Corresponding authors at: College of Fisheries, Huazhong Agricultural University, Wuhan, 430070, China.
E-mail addresses: sunqian90512@126.com (Q. Sun), cliu@mail.hzau.edu.cn (C. Liu).

https://doi.org/10.1016/j.aquatox.2020.105477
Received 27 August 2019; Received in revised form 19 March 2020; Accepted 21 March 2020
Available online 26 March 2020
0166-445X/

room and a kindergarten playing room were 9 and 3 ng/m^3, respectively (Tollbäck et al., 2006). Besides, TCEP was widely distributed in surface water around the world. The mean concentration of TCEP in Spanish rivers was 85 ± 10 ng/L (García-López et al., 2010). In the River Ruhr (Germany), concentrations of TCEP ranged from 13 to 130 ng/L (Andresen et al., 2004), while the greatest concentration observed was 130 ng/L in the River Schwechat (Austria) (Martínez-Carballo et al., 2007). Concentrations of TCEP in Dongbok and Paldang Lakes, Korea, which are reservoirs that are sources of drinking-water, were 14 and 25 ng TCEP/L, respectively (Kim et al., 2007), and concentrations were less than those in drinking water (99 ng/L) in the United States (Stackelberg et al., 2004). Concentrations of TCEP in drinking water were comparable with those in natural waters. These results suggested that TCEP was difficult to remove during treatment of drinking water. Results of previous studies showed that in sediments from Norway, concentrations of TCEP ranged from 0.16 to 8.5 μg/kg wet mass (wm) (Leonards et al., 2011), and concentration of TCEP in sediments from Schwechat, Austria was 160 μg/kg dry mass (dm) (Martínez-Carballo et al., 2007). Furthermore, mean concentrations of TCEP in dust collected from cars, homes and offices in Germany were 950, 200 and 120 ng/g, respectively (Brommer et al., 2012).

TCEP is not only distributed in abiotic matrices, but also accumulated into biota, which suggests potential hazard to wildlife. In previous studies, TCEP was detected in aquatic organisms. For instance, in cod liver and mussels from Norway, the concentrations were less than 5 and 10–23 μg TCEP/kg wm, respectively (Green et al., 2008). Concentrations of TCEP in 24 samples of fishes from Italy were less than 0.4 μg TCEP/kg wm (Campone et al., 2010). In Norway, concentrations of TCEP in blue mussels were less than 0.06 μg TCEP/kg wm, however, in liver of burbot (*Lota lota*) a concentration of 8.6 μg TCEP/kg wm was observed (Leonards et al., 2011). TCEP has also been observed in tissues of humans. For instance, in Sweden human breast milk, contained concentrations ranging from 2.1 to 8.2 ng TCEP/g (Sundkvist et al., 2010), while concentrations of TCEP in blood serum collected from Chinese people were between 248.6 and 958.2 ng/g lipid (Li et al., 2015b). Therefore, TCEP in the ambient environment might also pose a threat to health of humans.

Results of previous studies have shown that exposure to TCEP results in a range of effects, such as neurotoxicity, developmental toxicity and reproductive toxicity (Xu et al., 2017; Li et al., 2019; World Health Organization, 1998; Matthews et al., 1993; Chapin et al., 1997; Lehner et al., 2010; Ren et al., 2008). For example, it was reported that TCEP could cause locomotor deficits and dopaminergic degeneration in *Caenorhabditis elegans* and adversely affect neurodevelopment of zebrafish embryos/larvae (Xu et al., 2017; Li et al., 2019). Moreover, deformities of vertebrae were observed in killifish after exposure to 200 mg TCEP/L for 72 h (World Health Organization, 1998). In mammals, TCEP could affect fertilities of rats and mice (Matthews et al., 1993; Chapin et al., 1997). Acute lethality of dogs was caused by ingestion of car seat cushions containing a large amount of TCEP (Lehner et al., 2010). Results of in vitro experiments indicated that TCEP could inhibit proliferation of cells. For example, exposure to 10 mg TCEP/L in primary cultured, rabbit, renal proximal tubule cells, resulted in a reduction in the number of cells by down-regulating expression of cell cycle regulatory proteins and inhibiting synthesis of DNA (Ren et al., 2008). However, most of these studies focused on assessment of TCEP using higher tropic level organisms, while little is known about the effects of TCEP on lower tropic level organisms. Lower tropic level organisms represent an important link of energy flow and material circulation in food webs (Deng et al., 2015). Because fluctuations of populations of lower tropic level organisms might affect higher tropic level organisms, it is necessary to evaluate effects of TCEP on lower tropic level organisms.

In this study, effects of TCEP on growth and reproduction of the protozoan, *T. thermophila*, were evaluated, then transcriptomic sequencing technology was utilized to explore potential molecular mechanisms of toxic responses.

2. Materials and methods

2.1. Chemicals and reagents

Tris (2-chlorethyl) phosphate (TCEP, purity ≥99.0 %), penicillin G, streptomycin sulfate and amphotericin B were obtained from Sigma-Aldrich (St. Louis, MO, USA). The stock solution of TCEP was dissolved in dimethyl sulfoxide (DMSO), and both exposure and control groups received 0.1 % DMSO. TRIzol reagent, reverse transcription and SYBR Green kits were purchased from Takara (Dalian, Liaoning, China). Proteosepeptone, yeast extract, ethylendiaminetetraacetic acid monosodium ferric salt (Fe-EDTA) and D (+) glucose were from BactoDifco (USA), Oxioid Ltd (Basingstoke Hampshire, England), Aladdin (Shanghai, China) and Biosharp (USA), respectively. All reagents used in this study were of analytical grade.

2.2. Culture of T. thermophila

The SB210 strain of *T. thermophila* was obtained from the Tetrahymena Stock Centre (Cornell University, New York, USA). As described before, *T. thermophila* cells were cultured in super proteose peptone (SPP) medium, which contained 2 % proteose peptone, 0.1 % yeast extract, 0.2 % D (+) glucose, 0.003 % Fe-EDTA, 100 units/mL penicillin G, 100 mg/L streptomycin sulfate and 0.025 mg/L amphotericin B (Li et al., 2015a).

2.3. TCEP exposure protocol

Exposure to TCEP was carried out in two stages. Exposure concentrations were selected based on the results of our preliminary experiments. During the first stage, *T. thermophila* cells were inoculated in a 24-well plate at a density of 2×10^4 cells/mL and exposed to 0, 1, 2, 4, 8, 16, 32 or 64 mg TCEP/L for 18 h. Numbers and viability of cells were measured and half maximal inhibitory concentration (IC_{50}) was calculated to determine acute toxicity of TCEP. During the second stage, cells were inoculated at a seeding density of 2×10^4 cells/mL and then exposed to 0, 0.05 mg/L (1/200 of IC_{50}), 0.5 mg/L (1/20 of IC_{50}) and 5 mg/L (1/2 of IC_{50}) TCEP for 24 h. Thereafter, exposed cells or unexposed controls were inoculated into new exposure medium or control medium also at a seeding density of 2×10^4 cells/mL and exposed for another 24 h. This exposure step was repeated until the end of the exposure period of 5 days. After five days exposure, cells were then collected to examine the effects of TCEP on body size and gene transcription as described previously (Li et al., 2015a). Briefly, cells were fixed in 4% paraformaldehyde and photographed by use of a Leica microscope (Leica DMI6000B). Body length, body width, and circumference of the cells were measured by the use of the ImageJ software. During the exposure, numbers of cells before inoculation were measured using a cell counter (Mini-006-0565, Nexcelom Bioscience, LLC., Lawrence, MA), then every 24 h, cells were inoculated into new medium for culture. The theoretical population, which includes cumulative growth of the population, was calculated by use of a previously published method (Eq. (1)) (Li et al., 2016). Each concentration included three replicated wells.

$$Pn = Dn \times Vn \times \prod_{k=0}^{n-1} \frac{Dk}{2 \times 10^4} \quad (1)$$

Where: P is theoretical population; D is the density of *T. thermophila*; V is the volume of medium; n is the day when theoretical population is calculated (n ≥ 1).

2.4. Quantification of TCEP in culture medium and cells

TCEP was extracted from culture medium by liquid - liquid extraction (LLE). Briefly, 1 mL medium (no cells) was transferred to a test tube and then 20 μL internal standard was added. Then, 1 mL HEX (n-hexane)/DCM (dichloromethane) (1:1) was added and vortex mixed for 2 min. The mixture was centrifuged at 1556*g* for 10 min and the upper organic phase was eluted to a new test tube. The extraction was repeated twice and collected organic phases were combined. Afterwards, the pooled organic phase was evaporated to dryness with nitrogen, and the residue was dissolved in 1 mL MeOH for instrumental analysis.

After exposure, cells were collected by centrifugation, and then washed once with fresh medium without TCEP and collected again. After that, cells were lyophilized. Eight milligrams dry mass of lyophilized cells was weighed and placed into a test tube, and 20 μL internal standard was added. After 30 min, cells were extracted with 1 mL HEX /DCM (1:1) three times and the organic phases were combined, and evaporated to dryness with nitrogen. Concentrated extracts were diluted in 1 mL MeOH and further purified with 300 mg of primary secondary amine (PSA). After vortexing (1 min) and centrifugation (2432*g*, 10 min), the supernatant was collected for instrumental analysis. Three replicates (independent samples) were performed measured for each concentration.

Samples were diluted to be within a predetermined calibration range and electrospray ionization (ESI(+)) was used in the multiple reaction monitoring (MRM) mode in a Waters ACQUITY UPLC® H-Plus Class system (UHPLC) coupled to a Waters® XevosTM TQ-XS mass spectrometer (TQ-XS/MS) (Milford, MA, USA). LC was separated on an ACQUITY UPLC® C18 column (2.1 mm × 50 mm, 1.7 μm particle size) (Waters Made in Ireland). Methanol (A) and 5 mM aqueous ammonium acetate (B) were the mobile phase of LC. The mobile phase flow rate was 0.3 mL/min and the gradient was set as follows: 0 min, 5 % A; maintained for 0.5 min; 0.5–7.5 min, 100 % A (linear); maintained for 2 min; 9.5–10 min, 5 % A (Linear); 10–12 min, 5 % A. The desolvation temperature was 400 °C and the capillary voltage was 0.8 kV. The cone gas flow rates and desolvation gas were 150 and 800 L/h, respectively. Mean recovery of TCEP was 91 ± 11 % for medium and 88 ± 7 % for cells. The limit of quantification (LOQ; (10 × S/N)) of TCEP in medium and cells was 50 ng/L and 6.25 ng/g dm, respectively.

2.5. Cell viability assay

Cell viability was determined using Cell Counting Kit-8 (CCK-8) (Biosharp, Wuhan, China). Briefly, after exposure, 100 μL cell suspension was transferred to a 96-well plate (Corning Costa, Corning, NY, USA). After that, 10 μL CCK-8 were added into each well and the plate was cultured for 3 h (135 rpm at 30 °C). Then, absorbance was measured at 450 nm by microplate reader (BioTek Instruments, Inc, Beijing).

2.6. Transcriptomic sequencing

2.6.1. Isolation and sequencing of RNA

To explore molecular mechanisms of toxic effects of TCEP, the control group and the greatest concentration group (5 mg/L) of *T. thermophila* cells were collected for transcriptomic analysis. For the control and 5 mg/L exposure group, each contained three replicate wells. Transcriptomic sequencing was accomplished by Novogene Bioinformatics Technology Co., Ltd. (Beijing, China) (Li et al., 2017). In summary, total RNA was isolated with TRIzol reagent (Invitrogen, New Jersey, NJ, USA). Quality and concentrations of RNA were determined by use of an Agilent 2100 Bioanalyzer system and Qubit® RNA Assay Kit, respectively. Concentrations of RNA ranged from 1 to 2 ug/uL and the ratio OD260/OD280 ranged from 1.95 to 2.05. Purified, high-quality RNA (RIN > 7) was used to construct the final cDNA library using NEBNext® UltraTM RNA Library Prep Kit (Illumina) and sequenced on the Illumina Hiseq 2500 platform.

2.6.2. Sequence tag preprocessing and mapping

Original image data collected by the high-throughput sequencer was converted into sequence data by use of CASAVA base recognition and saved as FASTQ files. In order to ensure quality and reliability of data analysis, original data was filtered to remove reads with adapters and low-quality reads. Finally, at a sequencing depth of 60×, about 50 million clean reads were acquired from each of six libraries, of which more than 92 % were mapped to the Tetrahymena Functional Genomic Database (http://tfgd.ihb.ac.cn/).

2.6.3. Expressions of genes calculation and pathway analysis

Expressions of genes were normalized by the expected numbers of fragments per kilobase of transcript sequence per millions base pairs sequenced (FPKM). In the present study, differentially expressed genes were filtered, based on fold change > 2 and *P*adj value (FDR) < 0.05 (Cheng et al., 2019). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was implemented using KOBAS (2.0) and enriched pathways were determined by corrected *P*adj value (FDR) ≤ 0.05.

2.7. qRT-PCR reaction validation

Transcriptomic sequencing revealed that SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) interactions in vesicular transport, proteasome and endocytosis pathways were significantly enriched. 15 genes were selected randomly from each of these three pathways for qRT-PCR validation (Zhu et al., 2015; Cheng et al., 2019). The qRT-PCR was performed according to previously reported methods (Zhu et al., 2015) and met requirements for minimum information for publication of quantitative real-time PCR experiment (MIQE, a set of guidelines that describe the minimum information necessary for evaluating qPCR experiments) guidelines (Bustin et al., 2009). Total RNA was extracted with TRIzol reagent (Takara, Dalian, Liaoning, China), and total concentrations of RNA were measured by use of an Epoch Microplate Spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA). First strand cDNA synthesis was accomplished by Prime Script™ RT reagent kits (Takara, Dalian, Liaoning, China) and qRT-PCR was conducted by use of SYBR Green kits (Takara, Dalian, Liaoning, China). The specificity and purity of PCR products were evaluated by use of melting curves. Gene sequences were acquired from the Tetrahymena Functional Genomics Database (http://tfgd.ihb.ac.cn/). Primer sequences were engineered by NCBI online design tools (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Table 1). The expression of *kti12* (Kluveromyces lactis toxin insensitive protein 12) gene did not change after exposure to TCEP, so it was selected as the internal control or housekeeping gene. The qRT-PCR reaction conditions were 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 1 min, and 72 °C for 45 s. Gene expression was analyzed using the $2^{-\Delta\Delta CT}$ method and converted to fold change. Every concentration contained three replicates.

2.8. Statistical analyses

All data were analyzed by SPSS 22.0 software. Homogeneity of variance and normality of the data were determined using Levene's test and Kolmogorov-Smirnov test, respectively. Significant differences between the control and exposure groups were determined by one-way analysis of variance (ANOVA) and Tukey's multiple range test. IC values were calculated by use of probit regression in SPSS 22.0 software. $P < 0.05$ indicated a significant difference.

Table 1
Sequences of primers for genes studied by Q-PCR.

Gene Name	Gene ID	Efficiencies of primers (%)	Sense primer (5' to 3')	Antisense primer (5' to 3')
Kti12	TTHERM_01444910	94.6	GGCAGAAAAATTCGCAAGAGGA	AGCAGCTCTAGCAAGGCAAA
PreTt29909	TTHERM_00185870	95.0	TGTTTAGGAGTTCAACCCGGAG	GTTGAGGCTGTGCCGACTAA
PreTt02546	TTHERM_00248500	91.0	TGCCTCCTGAATGGGTCGAT	CGCCGAATCCTCTTTTCAGTC
PreTt06034	TTHERM_00335890	92.5	AGCTGAAGATCCAAACAGGGC	GCATCATTTTGTGCAACTCCA
3830.m00587	TTHERM_00532799	95.1	TCATAAGGGCGCTGAAGCTG	ACCTCATCTTGCTTTTCTCCCA
PreTt20897	TTHERM_00314870	90.0	TTGTGGGCATTCAAAAGGGT	GTGAATGCGTATGCAACAACA
PRE7	TTHERM_00470650	91.5	AAAAGTACGCCGTCTTGGGA	AGCTGATTCAAAGCAATCGACA
RPN9	TTHERM_00388440	94.6	TTCCTTGCTTACACCCAACC	GTGTACAACCATGAGTAAGTAGTGT
PUP2	TTHERM_00043880	91.0	CCCATGAGTGTCCGCTCTTT	ACACCAAAAGGTCTGGGCAT
RPN15	TTHERM_00227230	95.6	AGTCCTCGACGAAGATGATGA	ATCGTCCCAATCTTCACGCC
PRE4	TTHERM_00548160	91.5	GAACGTCATCGGAGGTGTTG	GAGAGGTCCGCAAAGGTAGT
PreTt28977	TTHERM_00576900	105.9	AGTTGGTGCTGGAGTAGTGC	GCAGCAAGGCTAGTTACCTCA
SSA4	TTHERM_01080440	90.7	ACAGCAGAAGCATTCGTTTCC	ATAGCTGCAGCAGTAGGCTC
PreTt12293	TTHERM_00856680	92.7	TAGGGCGCAAAAGTGATTGG	TAGCGCACAAGCCTTCAAAC
RAB40	TTHERM_00066880	96.2	TTGGGATACTGCTGGCTAGG	GGAGAATTCTACATGCTTCTGATCC
PreTt10217	TTHERM_00079720	94.1	ACACTCTTGAAATGGGTACTGC	GCGAATTCTTCATCAAGTTCAGC

3. Results and discussion

3.1. Exposure to TCEP caused acute toxicity

Exposure to TCEP for 18 h caused a dose-dependent inhibition of proliferation on *T. thermophila* (Fig. 1). Exposure to 8, 16, 32 or 64 mg TCEP/L for 18 h significantly decreased numbers of cells, but exposure to lesser concentrations of 1, 2 or 4 mg TCEP/L did not result in statistically significant differences from controls. Viabilities of cells were significantly less after exposure to 4, 8, 16, 32 or 64 mg TCEP/L for 18 h, while no significant effects relative to controls were observed for cells exposed to 1 or 2 mg TCEP/L. When numbers of cells were used as an integrative index of effects, the 20% inhibitory concentration (IC_{20}) was 4.56 mg TCEP/L, and the calculated IC_{50} of TCEP was 11.85 mg TCEP/L (Fig. 1A). However, based on cell viability, the IC_{20} was 4.38 mg TCEP/L and the IC_{50} was 9.86 mg TCEP/L (Fig. 1B). In previous studies, 96h-LC_{50}s of TCEP to goldfish (*Carassius auratus*) and killifish (*Oryzias latipes*) were 90 and 210 mg/L, respectively (Sasaki et al., 1981), while for rainbow trout (*Oncorhynchus mykiss*) the 96h-LC_{50} was 249 mg/L (World Health Organization, 1998). For the cladoceran, *Daphnia magna*, the LC_{50} of TCEP was > 100 mg/L after 24-h exposure (World Health Organization, 2009). For the nematode, *Caenorhabditis elegans*, the LC_{50} after exposure to TCEP for 1, 3 or 6 days were 1825 mg/L, 1578 mg/L and 852 mg/L, respectively (Xu et al., 2017). Therefore, these results suggested that *T. thermophila* was more sensitive to TCEP than were other species, although these organisms have much greater body size which might affect accumulation of TCEP. These results also indicated that *T. thermophila* was more suitable for acute toxicity studies of TCEP than other species mentioned above (*Daphnia magna*, etc.). It should be noted that toxic effects of TCEP observed in this study occurred at concentrations in the mg/L range, which exceeded environmental concentrations.

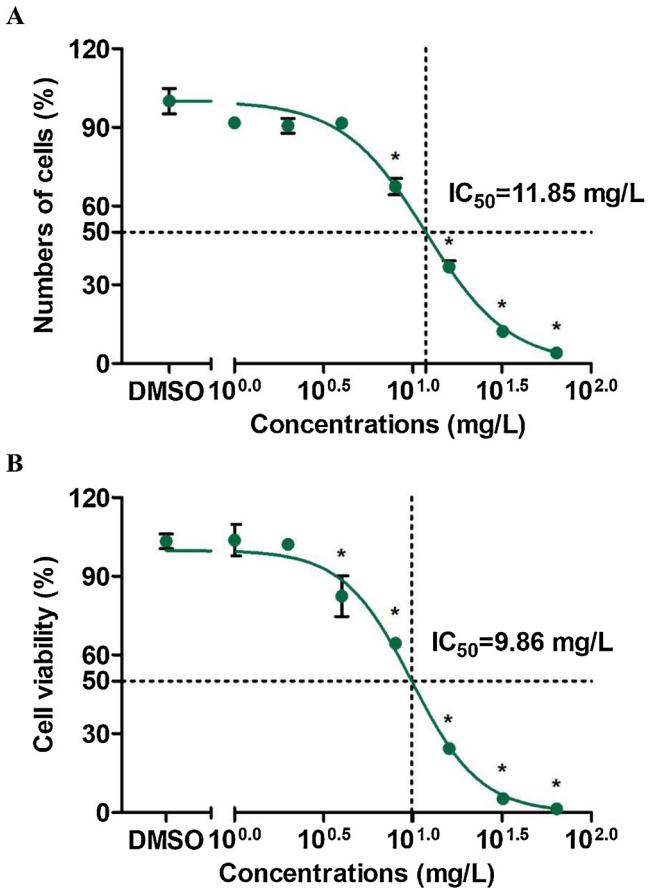

Fig. 1. Dose-dependent effects on numbers of cells and cell viability of *T. thermophila* after exposure to 0, 1, 2, 4, 8, 16, 32 or 64 mg TCEP/L for 18 h. Concentrations were log_{10}-transformed and the values of both endpoints were fitted to nonlinear regression curve (log_{10} (inhibitor) vs. normalized response) to determine the half maximal inhibitory concentration (IC_{50}).Values represent mean ± SD (n = 3). Significant differences are indicated by $^*P < 0.05$.

3.2. Exposure to TCEP reduced theoretical population, cell viability and numbers of cilia

In exposure medium, nominal concentrations of TCEP were 0.05, 0.5, and 5 mg/L, while measured concentrations of TCEP after medium renewing were 0.044 ± 0.000, 0.411 ± 0.003 and 4.26 ± 0.177 mg TCEP/L, respectively. When measured before renewing the medium, concentrations were 0.043 ± 0.002, 0.390 ± 0.018 and 4.05 ± 0.174 mg TCEP/L, respectively (Fig. 2A). Therefore, these data indicated that TCEP was not significantly metabolized or removed in this exposure system with *T. thermophila*. Mean concentrations of TCEP in cells were $4.22 \times 10^2 \pm 2.16 \times 10^1$, $2.93 \times 10^3 \pm 5.80 \times 10^1$ and $2.34 \times 10^4 \pm 6.39 \times 10^2$ ng TCEP/g dm when exposed to concentrations of 0.044 ± 0.000, 0.411 ± 0.003 or 4.26 ± 0.177 mg TCEP/L, respectively (Fig. 2B).

After exposure to 0.044, 0.411 or 4.26 mg TCEP/L, the theoretical population was decreased in a time-dependent manner and the significant difference was initially observed after four days of exposure (Fig. 3). Cell viability did not change significantly when exposed to 0.044 or 0.411 mg TCEP/L. However, when exposed to 4.26 mg TCEP/L, viability of cells was significantly less by 13.84 % compared to the control (Fig. 4). Numbers of cilia of *T. thermophila* decreased in a dose-

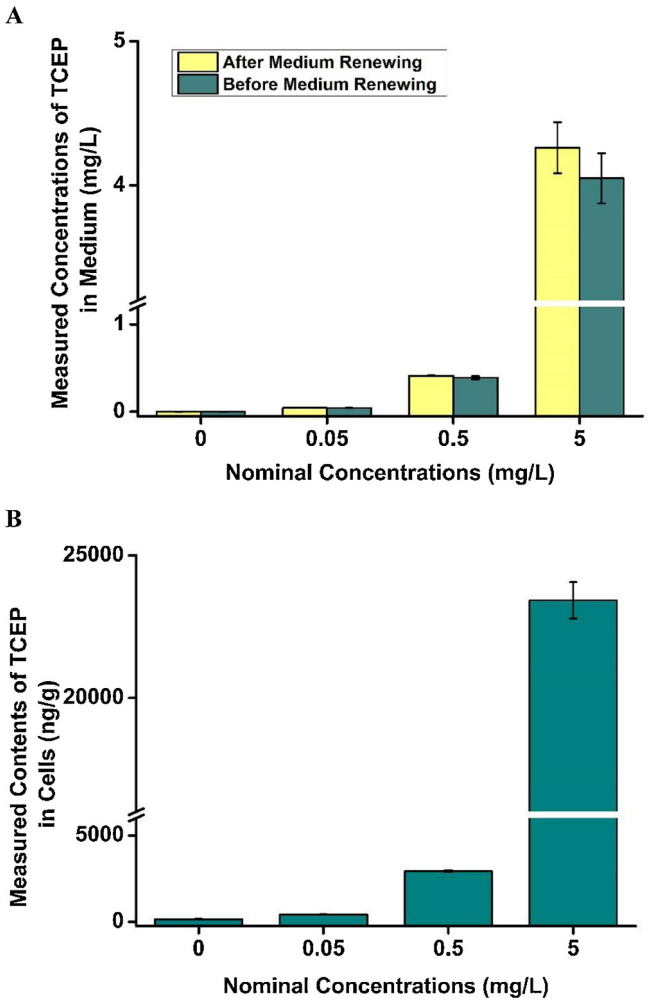

Fig. 2. Measured concentrations of TCEP in the culture medium samples without cells (mg/L) (A) and cell samples (ng/g dm) (B). Values represent mean ± SD (n = 3).

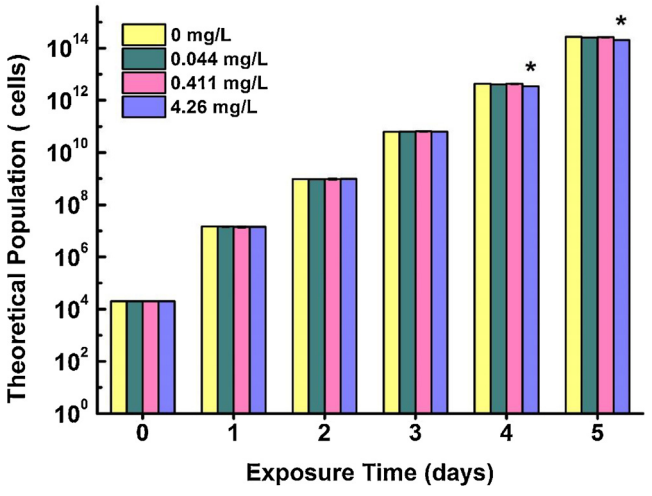

Fig. 3. Time-dependent effects on theoretical population of *T. thermophila* after exposure to 0, 0.044, 0.411 or 4.26 mg TCEP/L for 1, 2, 3, 4 or 5 days. Values represent mean ± SD (n = 3). Significant differences are indicated by $*P < 0.05$.

dependent manner, and they were significantly decreased by 12.8 % and 20.1 % when exposed to 0.411 or 4.26 mg TCEP/L (Fig. 5).

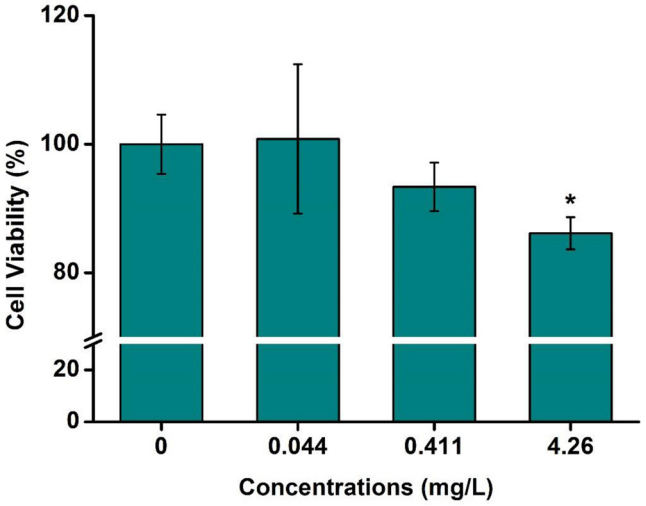

Fig. 4. Effects on cell viability of *T. thermophila* after exposure to 0, 0.044, 0.411 or 4.26 mg TCEP/L for 5 days. Values represent mean ± SD (n = 3). Significant differences are indicated by $*P < 0.05$.

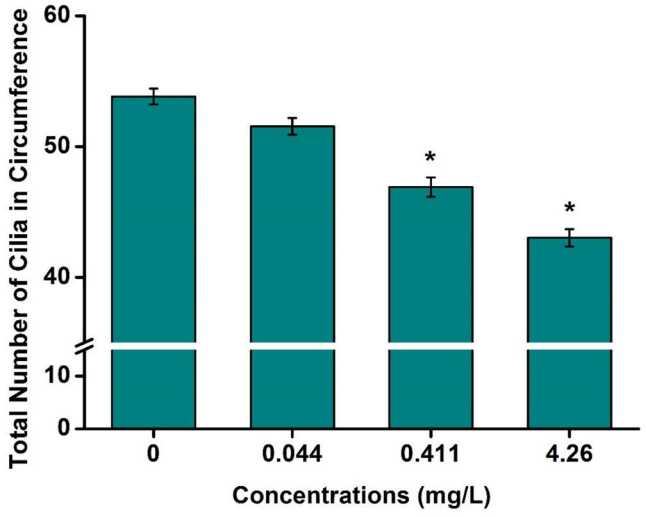

Fig. 5. Dose-dependent effects on cilia quantity of *T. thermophila* after exposure to 0, 0.044, 0.411 or 4.26 mg TCEP/L for 5 days. Values represent mean ± SD (n = 30). Significant differences are indicated by $*P < 0.05$.

Viability of cells is strongly related to the life activity of *T. thermophila*, and cilia are organelles that are responsible for sensory perception and individual movement in protozoa (Mitchell, 2007). Therefore, changes in these phenotypes might affect individual activity and population abundance. Consistent with the present study, Chen et al. (2015b) observed that exposure to 300 mg TCEP/L reduced cell viability, induced oxidative stress and disrupted steroidogenesis in murine Leydig cell line (TM3). In rat pheochromocytoma cell line (PC12), TCEP (43.0 and 57.3 mg/L) was observed to cause lesser viability of cells, increase cell apoptosis and change cell morphology (Ta et al., 2014). In addition, treatment with 10 mg TCEP/L significantly decreased the number of primary cultured rabbit renal proximal tubule cells by 30% compared to the control (Ren et al., 2008). Taken together, results from the present study and previous studies suggested that at relatively great concentrations (mg/L) TCEP caused cytotoxicity and inhibited proliferation of cells.

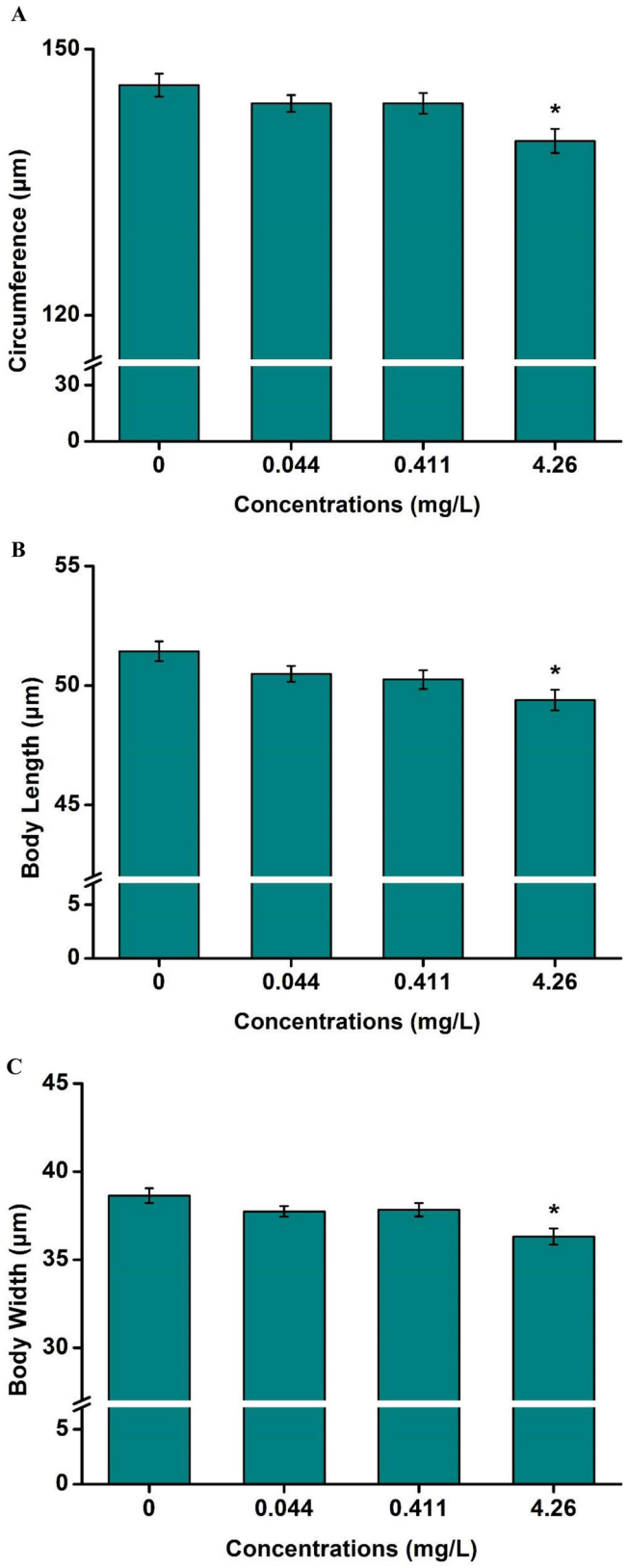

Fig. 6. Effects on circumference (A), body length (B) and body width (C) of *T. thermophila* after exposure to 0, 0.044, 0.411 or 4.26 mg TCEP/L for 5 days. Values represent mean ± SD (n = 250). Significant differences are indicated by $*P < 0.05$.

3.3. Exposure to TCEP decreased circumference, body length and body width

Exposure to the greatest concentration of 4.26 mg TCEP/L for 5 days significantly reduced mean circumference of *T. thermophila* by 4.3 %, while exposure to 0.044 or 0.411 mg TCEP/L did not significantly reduce mean circumference (Fig. 6A). In the control group, the mean body length and body width of *T. thermophila* were 51.4 and 38.6 μm, respectively. However, mean body length and body width of *T. thermophila* were significantly lessened (49.4 and 36.3 μm) after exposed to 4.26 mg TCEP/L (Fig. 6B and C). These results were consistent with the findings of previous studies (Ta et al., 2014; Chen et al., 2015a; Xu et al., 2017). In *C. elegans*, body length, body width and average length of life were significantly shortened after exposure to 750 or 1000 mg TCEP/L (Xu et al., 2017). Masses of the body, liver and testis of male mice were significantly reduced after exposure to TCEP (300 mg/kg wm) (Chen et al., 2015a). Morphology of PC12 cells was changed following exposure to 43.0 and 57.3 mg TCEP/L (Ta et al., 2014). Results of these studies showed that TCEP had individual-level toxic effects on the growth or reproduction of organisms by affecting the body length, body width, mass and morphology of individuals. These effects on individuals might further cause population-level responses. However, it should be noted that the toxic effects of TCEP in this study occurred in the order of mg/L, which exceeded environmental concentrations. Therefore, further studies are needed to evaluate effects of TCEP after exposure to more environmentally relevant concentrations.

3.4. Transcriptomic responses revealed possible molecular mechanisms of TCEP toxicity

In this study, using the *P*adj value < 0.05 as a standard, compared with the control group, expressions of 2932 genes in *T. thermophila* exposed to 4.26 mg TCEP/L were significantly changed, with 1228 up-regulated and 1704 down-regulated. The 2932 genes were further used for KEGG pathway analysis and SNARE interactions in vesicular transport pathway (9 genes, down-regulation), proteasome pathway (10 genes, down-regulation) and endocytosis pathway (17 genes, down-regulation) were significantly enriched (Fig. 7). Transcriptomics data indicated that the SNARE interactions in vesicular transport, proteasome, and endocytosis pathways were significantly affected. The reason why the only 36 genes were enriched in KEGG pathway analysis might be that the gene annotation of *T. thermophila* was not very accurate, which resulted in that some genes were not enriched in any pathway. In our previous study (Cheng et al., 2019), we also found that although 6600 differentially expressed genes were obtained, but only 30 genes were enriched in KEGG pathway analysis.

Vesicular transport provides proteins and lipids required for many important life activities of eukaryotic cells, such as the formation and maintenance of eukaryotic organelles, protein targeted transport, neurotransmitter release and cell growth (Zhao et al., 2012). SNAREs are the core machinery that mediates membrane fusion and membrane fusion is the last step in vesicular transport (Wang et al., 2017). Therefore, down-regulation of genes involved in protein metabolism and nutrient transport observed during this study might be responsible for the decrease of body length, body width and circumference. Endocytosis is a process by which cells bring extracellular material and plasma membranes into the interior of cells (Dutta and Donaldson, 2012) and it important for regulation of life cycles and metabolism of cells (Silverstein et al., 1977). Down-regulation of endocytosis protein genes might inhibit nutrient acquisition, life cycle and metabolism of cells, associated with inadequate absorption and utilization of nutrients, and thereby affect growth and reproduction of cells, such as the observed decrease in cell viability. The proteasome is a rich multi-enzyme complex. In eukaryotic cells, the proteasome is the site of most cellular protein degradation and essential for survival, which was the key to intracellular protein degradation, and its orderly degradation

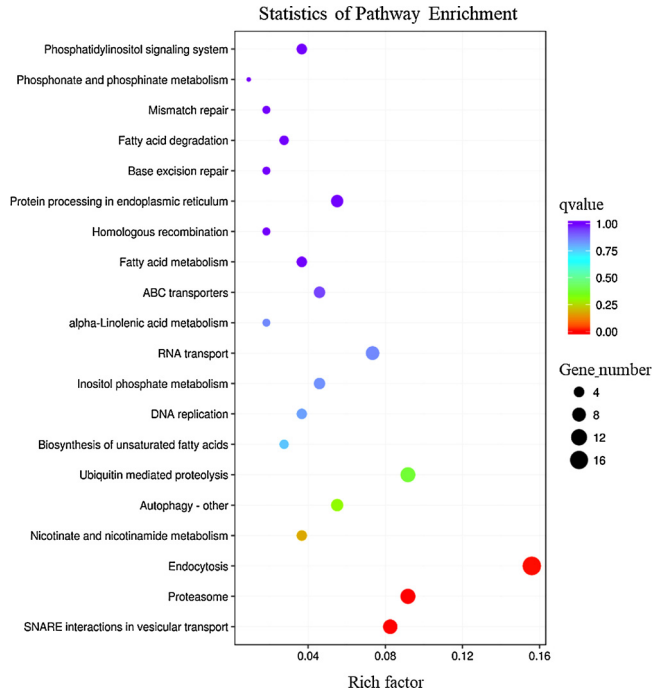

Fig. 7. Significantly enriched KEGG terms. The different colors indicate different *p*adj values, and the different circle sizes indicate different number of genes included in the corresponding pathways.

was essential for maintaining normal cellular function (Rock et al., 1994; Hilt et al., 1993; Tanaka, 1995). At the same time, it also plays a key role in regulation of the progression of the cell cycle (Adams, 2004; Leblanc et al., 2002; Hideshima et al., 2001; Frankel et al., 2000; Shah et al., 2001; Adams et al., 1999; Maclaren et al., 2001). Proteasome dysfunction directly affected cell cycle regulators, thereby disrupting regulation of the cell cycle, growth regulation, and genetic stability (Livneh et al., 2016; Manasanch and Orlowski, 2017). Therefore, down-regulation of the proteasome protein genes might cause proteasome dysfunction, thereby disrupting the cell cycle and further inhibiting proliferation of cells, such as the observed reduction in the theoretical population. To verify the results of transcriptome sequencing, fifteen genes were randomly selected from the SNARE interactions in vesicular transport pathway, proteasome pathway and endocytosis pathway for qRT-PCR assays. The results showed that expression of these genes was dose-dependently altered after exposure to TCEP, consistent with RNA-Seq data (Fig. 8). Therefore, transcriptome sequencing results were deemed to be reliable. Results of the qRT-PCR validation showed that exposure to 0.411 mg/L TCEP significantly down-regulated expressions of genes involved in SNARE interactions in vesicular transport, proteasome and endocytosis pathways, but had no significant effects on cell viability, body size and population size. These results suggested that transcriptomic changes were more sensitive than were toxic endpoint parameters, and could be used to identify mechanisms of action of chemicals.

In summary, the results of this study suggested that greater concentrations of TCEP exposure (mg/L) could reduce the theoretical population, cell viability, cell size, and cilia number of *T. thermophila*. Also, molecular events revealed that cell growth and reproduction after exposure to TCEP was inhibited possibly through the down-regulation of vesicle trafficking, endocytosis, and proteasome protein genes. Therefore, results reported here provided evidence for the first time that TCEP was toxic to *T. thermophila* at relatively great concentrations, which exceeded environmental concentrations. Therefore, the results of this study suggested that in natural waters, exposure to TCEP might cause low environmental risk for *T. thermophila*.

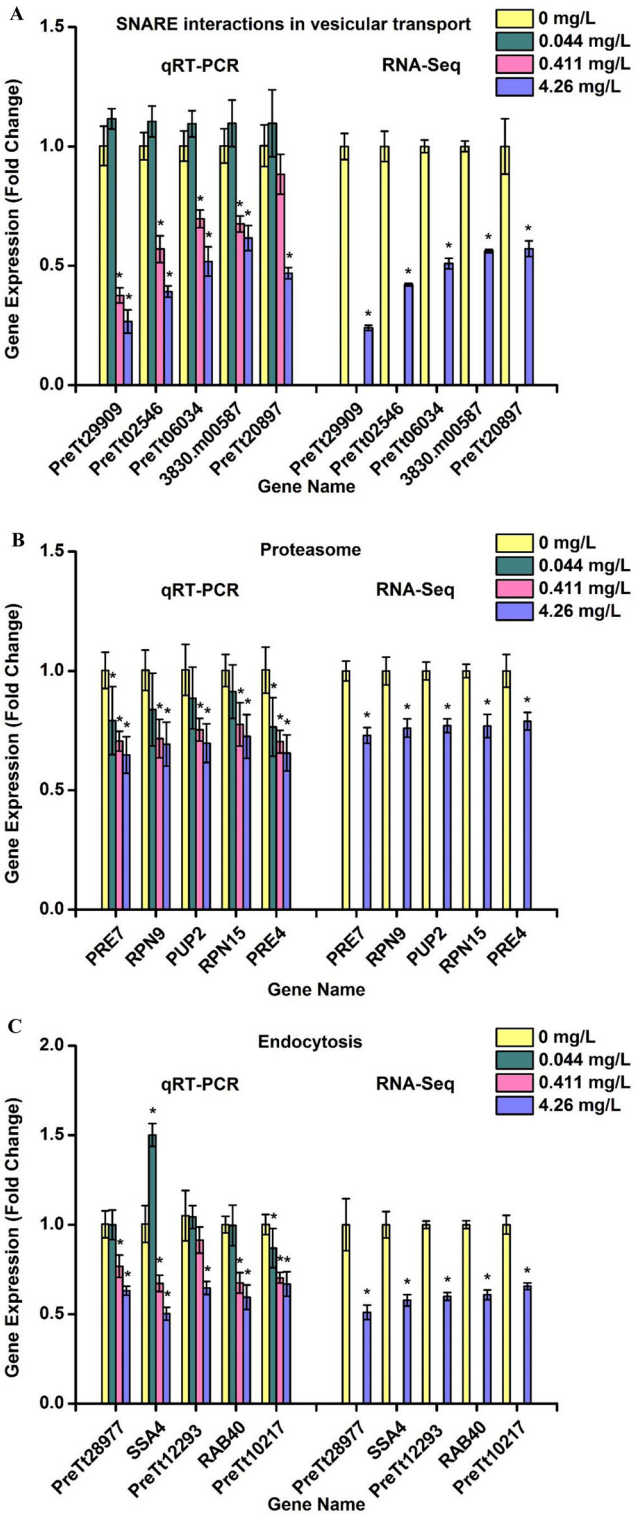

Fig. 8. Confirmation of RNA-Sequencing data with qRT-PCR. Values represent mean ± SD (n = 3). Significant differences are indicated by $^{*}P < 0.05$.

CRediT authorship contribution statement

Hui Hao: Formal analysis, Writing - original draft. **Siliang Yuan:** Visualization, Writing - review & editing. **Shiyang Cheng:** Investigation, Writing - review & editing. **Qian Sun:** Investigation. **John P. Giesy:** Writing - review & editing. **Chunsheng Liu:** Methodology, Project administration, Writing - review & editing.

Declaration of Competing Interest

There are no conflicts to declare.

Acknowledgements

This work was supported by National Key R&D Program of China (2017YFF0211203) and National Natural Science Foundation of China (21622702). Prof. Giesy was supported by the Canada Research Chairs Program of the Natural Science and Engineering Research Council (NSERC) of Canada and a Distinguished, Visiting Professorship from Baylor University.

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